The telomerase reverse transcriptase regulates chromatin state and DNA damage responses

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Constitutive expression of telomerase prevents senescence and crisis by maintaining telomere homeostasis. However, recent evidence suggests that telomerase is dynamically regulated in normal cells and also contributes to transformation independently of net telomere elongation. Here, we show that suppression of the telomerase catalytic subunit [human telomerase reverse transcriptase (hTERT)] expression abrogates the cellular response to DNA double strand breaks. Loss of hTERT does not alter short-term telomere integrity but instead affects the overall configuration of chromatin. Cells lacking hTERT exhibit increased radiosensitivity, diminished capacity for DNA repair, and fragmented chromosomes, demonstrating that loss of hTERT impairs the DNA damage response.

Normal human cells exhibit a limited replicative lifespan and eventually enter a growth arrest state, termed replicative senescence, triggered by dysfunctional telomeres (1). However, other stimuli such as oncogene activation (2), increased oxidative potential (3), and genotoxic damage (4) also trigger a cell cycle arrest that shares both morphologic and functional similarities with replicative senescence. Thus, although telomere maintenance plays an important role in regulating the proliferative potential of human cells, the role(s) of telomere biology in replicative senescence induced by successive cycles of cell division and in the senescence-like growth arrest state triggered by other stimuli remains obscure.

Moreover, recent work indicates that senescent human cells show evidence of activation of the DNA damage response pathway (5). Although overexpression of telomerase maintains telomere length and facilitates human cell immortalization (6, 7), accumulating evidence also suggests that telomerase itself plays an additional role in protecting karyotypic stability by “capping” chromosomes (8). Indeed, constitutive overexpression of telomerase reverse transcriptase (TERT) facilitates malignant transformation independently of its effects on overall telomere length (9) and renders cells more resistant to apoptosis (10). Because these observations connect telomerase expression, DNA damage responses, and senescence, we reasoned that human TERT (hTERT) may contribute to the cellular response to genotoxic insults. Here we show the effects of stably suppressing hTERT function in normal human fibroblasts on chromatin architecture and the response to DNA double strand breaks.

Materials and Methods

Stable Expression of Short Hairpin RNA (shRNA). The sequences shown in Table 2, which is published as supporting information on the PNAS web site, were introduced into the pMKO.1-puro vector (11) to create shRNA vectors specific for hTERT and GFP (Fig. S5, which is published as supporting information on the PNAS web site). These vectors were used to make high titer amphotropic retroviruses, which were used to infect human fibroblasts as described (11). Polyclonal cell populations were purified with selection with puromycin (2 µg/ml).

Immunoblotting, Immunofluorescence, FISH, and RT-PCR. For immunofluorescence, cells were fixed in chilled acetone, incubated with the primary antibody, washed, and then incubated with either an Alexa Fluor 568-conjugated or Alexa Fluor 488-conjugated secondary antibody (Pierce) in 1% BSA for 1 h at 37°C. For telomere-specific FISH, we hybridized a peptide nucleic acid (PNA) probe (CCCTAA)3 specific for mammalian telomeres (Applied Biosystems) to acetone-fixed cells at 72°C for 8 min. To remove nonhybridized PNA probes, slides were washed with 0.05% Tween 20 containing PBS at 56°C for 15 min and visualized by using a Nikon Eclipse E800 fluorescence microscope. We did not detect staining of parallel cultures with a single mismatch (CCCTTA)3. PNA probe. The antibodies used in this study were purchased from the following suppliers: rabbit anti-H2AX and mouse anti-hTERT (Novo Biologicals, Littleton, CO); rabbit anti-γ-H2AX, rabbit anti-H2B, mouse anti-H3, rabbit anti-H4, rabbit anti-macro H2A.1, rabbit anti-dimethyl H3 (K9), rabbit anti-acetyl H3 (Lys-9), and rabbit anti-acetyl H4 (K12) (Upstate Biotechnology); goat anti-phospho-specific breast cancer-associated 1 (BRCA1) (Ser-1497) (Santa Cruz Biotechnology); rabbit anti-ATM-pS1981 (Rockland, Gilbertsville, PA); and mouse anti-p53 (Ab6). (Oncogene Science). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (12.5 mM sodium phosphate, pH7.2/2 mM EDTA/50 mM NaF/1.25% Nonidet P-40/1.25% SDS/0.1 mM DTT) except when specific conditions are noted. For extraction under low salt conditions, cells were lysed in a buffer comprising 20 mM Tris-HCl, pH 7.4/150 mM NaCl/0.1% Nonidet P-40/0.1 mM DTT. For protein extraction under high salt conditions, 500 mM NaCl was substituted in the low salt buffer. For acid precipitation, cells were homogenized in 0.2 N H2SO4 and centrifuged. Histones were precipitated by adding 0.25× vol of 100% (wt/vol) trichloroacetic acid (TCA). The pellets were suspended in 100% ethanol and centrifuged at 13,000 × g. Ten micrograms of protein was subjected to immunoblotting. The sequences used for the H2AX RT-PCR were as follows: 5′-TCGGCGCAGGGCAAGACCTGGCCGCAA-3′ and 5′-GTACTCTGGAGGAGGCTTGA-3′. Reverse transcription was performed on 500 ng of total RNA for 30 min at 42°C, followed by PCR (25 cycles: 94°C for 45 s, 60°C for 45 s, and 72°C for 90 s).

Analysis of Telomere Structure. Telomere length was measured by hybridizing a 32P-labeled telomeric (CCCTAA)3 probe to HindIII- and RsaI-digested genomic DNA. Quantitative FISH (Q-FISH)

Abbreviations: Q-FISH, quantitative FISH; MN, micrococal nuclease; TERT, telomerase reverse transcriptase; hTERT, human TERT; DN hTERT, catalytically inactive hTERT mutant; shRNA, short hairpin RNA; ATM, ataxia–telangiectasia-mutated; BRCA1, breast cancer–associated 1; DAT, dissociates activities of telomerase; TSA, trichostatin A.
analysis was performed as described (12). Results of Q-FISH analysis are expressed in kilobases as determined by comparison with plasmid DNA containing telomere inserts. The telomeric 3' single-stranded overhang was analyzed by a telomeric 3' overhang assay (13).

**Micrococcal Nuclease Assay.** Cells (1 × 10⁶) were suspended in 1 ml of nucleic buffer [25 mM Hepes, pH 7.8/1.5 mM MgCl₂/10 mM KCl/0.1% Nonidet P-40/protease inhibitor mixture (Roche Applied Science, Indianapolis)]. Nuclei were obtained by Dounce homogenization (20 strokes, pestle A) and sedimented by centrifugation at 1400 × g at 4°C for 20 min through 1 ml of a solution containing 10 mM Tris- HCl, pH 7.4/15 mM NaCl/60 mM KCl/0.15 mM spermine/0.5 mM spermidine/10% sucrose. The nuclear pellet was then resuspended in 350 μl of digestion buffer (50 mM Tris-HCl, pH 7.5/15 mM NaCl/5 mM KCl/3 mM MgCl₂/1 mM CaCl₂/10 mM NaH₂SO₄/0.25 M sucrose/0.15 mM spermine/0.5 mM spermidine/0.15 mM mercaptoethanol) containing micrococcal nuclease (9 units/ml, Roche). Fifty microliters from this reaction mixture was mixed with 50 μl of stop solution (200 mM EDTA/200 mM EGTA, pH 7.5) to stop the reaction. Digested DNA was recovered by QIAquick columns (Qiagen, Valencia, CA), subjected to agarose gel electrophoresis, and visualized by staining with ethidium bromide. Direct application of digested chromatin without further purification to agarose gels gave similar results.

**Clonogenic Assay.** Clonogenic assays were performed by using two different seeding protocols. In some experiments, 200 cells were seeded into 9.6-cm² plates in triplicate and exposed to ionizing radiation after 24–48 h. Cells were allowed to proliferate for 10–12 days, trypsinized, and replated into plates to eliminate cell debris. Colonies were counted after an additional 5–7 days by using a Coulter particle counter. In other experiments, 1,000 cells were seeded into 9.6-cm² plates in triplicate, irradiated after 24–48 h, incubated 21 days, and stained with crystal violet (0.2%) to identify colonies. Colonies containing >20 cells were counted manually. Identical results were obtained by using these two methods, and the first method was used for the experiment shown in Fig. 4A.

**DNA Repair Assay.** The DNA repair assay was performed as described (14). Briefly, cells were mock irradiated or irradiated (2 Gy), allowed to recover at 37°C for 0, 2, and 4 h, trypsinized, and cast into 0.75% SeaPlaque agarose (FMC). These agarose gel plugs were placed in lysis buffer and incubated at 50°C for 30 min, washed with TE buffer, and equilibrated. The plugs were then subjected to pulse field gel electrophoresis in 0.7% agarose gels, dried, and stained with SYBR Green (Molecular Probes), and the fluorescence signal was measured by IMAGEQUANT software. The fraction of DNA entering the gel was determined by the following equation: ([signal in lane]/[signal in lane + signal in plug]) × 100. The relative fraction of DNA breaks repaired at 4 h was determined by calculating the ratio of DNA entering the gel at 4 h to that present immediately after irradiation (0 h). The measured value of signal present in unirradiated cells was subtracted for each sample. The data were normalized to the control shRNA sample and presented as bars representing the mean ± standard deviation.

**Cytogenetic Analyses.** Before or after exposure to 5 Gy of γ-radiation, cells were incubated at 37°C for 24 h and subjected to a standard cytogenetic protocol (15). Cytogenetic abnormalities were scored by a blinded observer.

**Results**

To assess the effects of suppressing hTERT function on the response to ionizing radiation, we examined well characterized changes in several proteins implicated in the response to DNA damage in diploid human fibroblasts, which only transiently express low levels of hTERT in S-phase (11). As expected, irradiation of human BJ or WI38 fibroblasts expressing a control, GFP-specific shRNA vector led to the phosphorylation of H2AX (γ-H2AX) (Fig. 1A, B, and E), to phosphorylation of the ataxia–telangiectasia-mutated (ATM) (Fig. 1C) and BRCA1 tumor suppressor proteins (Fig. 1D), and to the up-regulation of the p53 protein from basal levels (Fig. 1B and E). Treatment of these fibroblasts with irinotecan or etoposide, chemotherapeutic agents that induce DNA double-strand breaks, also induced phosphorylation of H2AX (Fig. 1D).
Surprisingly, exposure of parallel cultures of fibroblasts stably expressing either an hTERT-coding sequence-specific shRNA (hTERT shRNA) or an hTERT 3′ untranslated region-specific shRNA (hTERT 3′ UTR shRNA) (Fig. 5) to ionizing radiation, irinotecan, or etoposide failed to induce a similar degree of H2AX phosphorylation (Fig. 1 A, B, D, and E) or accumulation of Nijmegen Breakage Syndrome (NBS-1) in nuclear foci (data not shown). In addition, the autophosphorylation of ATM was diminished (Fig. 1C), and we failed to observe the phosphorylation of BRCA1 or the stabilization of p53 protein levels in cells lacking hTERT expression (Fig. 1 B and E). These findings indicate that the DNA damage response in cells lacking hTERT is impaired. Expression of WT hTERT, which is resistant to the effects of the hTERT 3′ UTR-specific shRNA (Fig. 5), in cells expressing this shRNA rescued telomerase activity (Fig. 2G) and permitted cells to respond to DNA damage (Fig. 1 B–D). We also found that fibroblasts expressing a catalytically inactive hTERT mutant (DN hTERT), which inhibits the catalytic activity of telomerase (16), also showed an impaired DNA damage response (Fig. 6, which is published as supporting information on the PNAS web site). Thus, chronic loss of hTERT function either by RNA interference or catalytic inhibition abrogates the cellular response to DNA damage, implicating hTERT as a critical regulator of the DNA damage response pathway.

Although overexpression of hTERT stabilizes telomere length in human cells (6), over the short time periods encompassed by these experiments, we did not detect alterations in overall telomere length (Fig. 2 A–C) or changes in the length of the 3′ telomeric single-stranded overhang (13) (Fig. 2D) either before or after irradiation of cells expressing an hTERT-specific shRNA as compared with cells expressing a control shRNA. Moreover, we noted that only 7% of nuclear foci containing γ-H2AX colocalized with telomeres after
Table 1. Statistical analysis of cytogenetic abnormalities

<table>
<thead>
<tr>
<th>Comparison group</th>
<th>Comparison values</th>
<th>P value</th>
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<tbody>
<tr>
<td>WT hTERT vs. vector control</td>
<td>0.524 vs. 0.718</td>
<td>0.41</td>
</tr>
<tr>
<td>Vector control vs. hTERT shRNA</td>
<td>0.718 vs. 1.31</td>
<td>0.02</td>
</tr>
<tr>
<td>WT hTERT vs. hTERT shRNA</td>
<td>0.524 vs. 1.31</td>
<td>0.008</td>
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Proportion of normal metaphases

| WT hTERT vs. vector control          | 0.619 vs. 0.462   | 0.25    |
| Vector control vs. hTERT shRNA       | 0.462 vs. 0.241   | 0.058   |
| WT hTERT vs. hTERT shRNA             | 0.619 vs. 0.241   | 0.008   |

Metaphase chromosomes were examined at 24 h after exposure to ionizing radiation (5 Gy) from BJ cells expressing a control vector, an hTERT-specific shRNA, or WT hTERT. P values were obtained by applying the Wilcoxon rank-sum test. Comparison values were calculated by dividing the number of the indicated findings by the number of metaphases examined. We also observed that total number of cytogenetic abnormalities found in hTERT shRNA-expressing cells was slightly increased when compared with vector control cells (P < 0.09). The number of metaphase cells examined was as follows: cells expressing WT hTERT (21 cells), cells expressing the hTERT-specific shRNA (29 cells), and cells expressing a control vector (40 cells). Examination of metaphases from unirradiated cells expressing the control shRNA (13 metaphases) or the hTERT-specific shRNA (15 metaphases) revealed no fragments.

To determine further whether the telomere elongation function of hTERT was required for the DNA damage response, we introduced several hTERT mutants into cells in which the endogenous hTERT was suppressed by the expression of the hTERT 3′ UTR-specific shRNA. Specifically, we expressed hTERT mutants that harbor mutations in the amino (N)- and carboxyl (C)-terminal DAT (disassociates activities of telomerase) domains (N-DAT92, N-DAT122, and C-DAT1127) as well as the DN hTERT mutant (Fig. 2E) (16, 19–21). These DAT mutants have previously been shown to reconstitute telomerase biochemical activity yet fail to elongate telomeres or to confer an immortal phenotype when expressed in human cells (19–21). We confirmed that these hTERT mutants exhibited telomerase activity (Fig. 2G) and failed to rescue the premature senescence phenotype found in human fibroblasts that lack endogenous hTERT expression (Fig. 2F) (11). Despite this defect in telomere maintenance, these hTERT mutants restored the ability of human fibroblasts to phosphorylate H2AX and stabilize p53 after exposure to ionizing radiation (Fig. 2G). We note that the N-DAT92 and C-DAT1127 only partially rescue the DNA repair response observed in senescent cells did not contribute to these experiments. Indeed, in unirradiated cells, we failed to identify evidence of karyotypic abnormalities in cells expressing either the control shRNA or an hTERT-specific shRNA before irradiation (See legend to Table 1), confirming that the suppression of hTERT in early passage fibroblasts does not, by itself, result in immediate telomere dysfunction.

Fig. 3. Suppressing hTERT expression alters chromatin state. (A) Effects of hTERT suppression on chromatin alterations induced by TSA. Cells were treated with TSA (10 μM) for 8 h. Phosphorylated ATM and total ATM protein levels were determined by immunoblotting. (B) MN digestion of nuclei derived from cells expressing the indicated shRNA vectors. Nuclei isolated from 1 × 10^6 cells were treated with MN for the indicated time, subjected to gel electrophoresis, and stained with ethidium bromide. The arrowhead indicates the migration of mononucleosomes. (C) Histone tail modifications. BJ cells expressing the indicated shRNA were lysed in radioimmunoprecipitation assay (RIPA), and immunoblotting was performed. (D) Extraction of H2AX from chromatin. BJ cells expressing the indicated shRNA were irradiated (10 Gy), incubated for the indicated time, and lysed with RIPA buffer, and immunoblotting was performed on whole cell lysates (100 μg). (E) H2AX mRNA expression. Total RNA (500 ng) was used for RT-PCR with primers specific for H2AX and β-actin. (F) Precipitation of H2AX from chromatin under acidic conditions from BJ cells expressing the indicated shRNA vectors. (G) Extraction of histones under low and high ionic strength. Cells were treated with 150 mM NaCl and 500 mM NaCl, and histones were extracted. (H) Extraction of core histones from chromatin. BJ cells expressing the indicated shRNA were lysed in RIPA buffer, and immunoblotting was performed on whole cell lysates (100 μg).
chronic suppression of hTERT expression not only alters the overall overexpressing hTERT (Fig. 3 consistently found slightly increased levels of H3 and H4 in cells hTERT expression (Fig. 3 contrast, we failed to detect differences in the amounts of soluble hTERT (Fig. 3 H2AX protein in whole cell lysates derived from cells lacking over a wide range of salt concentrations, we detected 75% less the result of altered H2AX expression (Fig. 3 fibroblasts expressing the control or either of the two chromatin structure and impaired DNA damage responses includ- ing recombination and nonhomologous end joining (26, 27). Because suppression of hTERT expression alters overall chromosome healing occurs much less frequently than occasionally repaired by telomere addition; however, this mecha- nism for chromosome healing occurs much less frequently than (28–30) and mammalian cells (31, 32) indicates that chromosome breaks at locations distinct from telomeres are occasionally repaired by telomere addition; however, this mechanism for chromosome healing occurs much less frequently than other forms of DNA repair. The observations presented herein implicate hTERT as a regulator of the DNA damage response pathway through its actions on chromatin structure. Surprisingly, hTERT seems to participate in chromatin maintenance in a manner different from its known role in telomere length maintenance. Although we failed to find evidence of significant telomere loss in
of chromatin plays a critical role in mammalian development (40), developmental compensation, as has been observed in mice lacking the retinoblastoma gene (41), may also occur in mice lacking mTert, masking the effects of germ-line mTert loss on chromatin structure and DNA damage. Interestingly, some investigators have reported that mice lacking one mTert allele maintain telomerases and exhibit increased genomic instability than control mice (42), suggesting that haploinsufficiency at the mTert locus impairs telomerase function.

In addition, despite harboring long telomeres and basal telomerase activity, murine tumors show evidence of increased telomerase activity (43). Because recent work in both transgenic mice and human cells suggests that increased TERT expression contributes to malignant transformation even in cells harboring long telomeres (9), the effects of hTERT on chromatin may provide a plausible mechanism for such additional functions of hTERT in maintaining chromosomal stability and suggest how TERT may contribute to cell transformation independently of its effects on telomere maintenance.

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